LESSON 20:
CULTURE AND REGENERATION OF PROTOPLASTS

Objective

The objective of this Lesson is to culture the protoplasts obtained through various isolation procedures (described in previous Lesson) and their regeneration which includes cell wall synthesis and development of callus. The callus so obtained is further cultured to produce a complete plant. The Lesson will also give students, information on the culture medium and other requirements for protoplast culture and regeneration.

Culture of Protoplasts

The first step in the protoplast culture is the development of a cell wall around the membrane of isolated protoplasts. This is followed by induction of divisions in the protoplast-derived new cell giving rise to a small cell colony. By manipulation of the nutritional and physiological conditions in the nutrient media, cell colonies may be induced to grow a callus continuously or to regenerate whole plants. The culture methods and requirements of isolated protoplasts are often similar to those of single cells

Isolated protoplasts or their hybrid cells are cultured either in a liquid or agar medium. The common practice of using a liquid culture medium includes either incubating protoplasts/heterokaryons in a thin layer or as small drops of nutrient medium inside a petri dish which, in turn, is covered by another petri plate and finally sealed with parafilm. The culture dish is then maintained at low-light or dark conditions at 25°-28°C.

For culturing protoplasts in the nutrient medium containing agar, the procedure given for Bergmann's cell plating technique may be followed. About 2 ml aliquots of isolated protoplasts of suitable density (103-105 cells ml-1) are mixed with an equal volume of agar nutrient medium, the temperature of which should not exceed 45°C. On solidification of agar, the culture plates are sealed and maintained in an inverted position at 25°-28°C. With this method, individual protoplasts or heterokaryons can be conveniently observed under a microscope and plating efficiency readily determined. Let it be noted, however, that some workers have found the plating protoplasts in a liquid nutrient medium on the top of a layer of solidified agar medium suitable for regeneration and further development of protoplasts.

I Nutritional Components

Protoplast culture media generally comprise nutrients similar to those required for callus and suspension cultures. However, the concentration of iron, zinc and ammonium used in plant tissue culture media may be too high for protoplast culture. Mostly the salts of B5 and MS media with some modifications have been found suitable. Increasing the calcium concentration in a protoplast culture medium, two to four times over the normal concentration of a cell culture medium may be beneficial in preserving membrane integrity (Torres 1989). Generally, protoplast culture media contain 3-5% sucrose but in some systems (tobacco protoplast cultures) a lower sugar (1.5%) content is required. Organic nitrogen in the form of CH is generally included in the medium as reduced nitrogen, and NH_4N0_3 (20 mmol 1^{-1}) as reduced inorganic nitrogen.

The vitamins used for protoplast culture are the same as used in standard tissue culture media. Both types of growth substances (auxins and cytokinins) are used in the protoplast culture media in various combination in order to induce cell wall formation and divisions in isolated protoplasts. Cereal protoplasts require 2,4-D which may be either sufficient (if used alone) or better in combination with cytokinin (Vasil and Vasil 1980). However, 2,4-D as the sole auxin source generally leads to loss of morphogenetic potential in the protoplast-derived callus. Other auxin sources are NAA or 1AA.The commonly used cytokinins are BAP, kinetin, 2iP, or zeatin. Although the exact combination of the two types of growth hormones in the medium, varies according to the species, it has been observed that protoplasts from actively growing cell cultures may find a high auxin/kinetin ratio suitable to induce divisions, while those derived from highly differentiated cells (leaf tissue) require a high kinetin/auxin ratio for regeneration.

II Osmoticum

During isolation and culture, protoplasts require osmotic protection until they regenerate a strong wall. Inclusion of an osmoticum in both isolation and culture media prevents rupture of protoplasts. A variety of ionic as well as non-ionic solutes have been tested for adjusting the osmotic potential of various solutions used in protoplast isolation and culture. The most widely used osmotica in a protoplast culture medium as well as in an enzyme mixture are sorbitol, mannitol, glucose, or sucrose. Protoplasts are more stable in a slightly hypertonic solution. For mesophyll protoplasts (cereals and/pea) sorbitol or mannitol may be a suitable osmotic stabiliser, whereas sucrose has been preferred over glucose or mannitol to culture protoplasts of potato, sweet pea, brome grass and cassava. For tobacco suspension cultures galactose and fructose have been used as osmotica. The osmolarity of the medium is gradually reduced by periodic addition of a few drops of fresh medium as soon as the protoplasts have regenerated walls and undergone divisions.

Ionic substances (335 $mmolL^{-1}$ KCI and 40 $mmolL^{-1}$ MgS04.7H2O) improve the viability of protoplasts and yield cleaner preparations. Usually enzyme solutions are supplemented with certain salts (5-100 $mmolL^{-1}$ $CaCl_2$) along with non-ionic osmotic stabilisers. Cocking and Peberdy (1974) developed a cell-protoplast washing (CPW) solution containing salts and a suitable osmoticum to provide stable and cleaner preparations. The CPW solution can be used during enzyme incubation and washing of protoplasts. The enzyme incubation period depends on its concentration in the solution and the type of material used.

Techniques Used For Culture

Multiple Drop Array (MDA) Screening

In 1977, Potrykus and co-workers developed the MDA technique for systematically screening a large number of multiple combinations of media constituents for protoplast culture. This technique was principally designed for cereal (maize) leaf protoplast cultures since they did not respond to culture regimes that regenerate protoplasts of solanaceous members. The MDA screening technique uses hanging droplets of 40 u1 as the experimental unit. Each droplet represents one combination of factors to be tested. The droplets are arranged in a regular array of 7 x 7 drops on the lid of a 9-cm petri dish. To test seven different auxins (factors) in combination with four different cytokinins in the medium, each of these factors is used in seven different concentrations. The whole experiment, therefore, includes 4 x 7 petri dishes. Since each petri dish contains 49 droplets this results in a total of 4 x 7 x 49 = 1372 two-factor combinations. This experiment can be performed by one person 5-6 hr in addition to the time required for media preparation, protoplast isolation and culture evaluation.

Plating Density

A density of 1 x 104 to 1 x 105 protoplasts/ml is optimal, since at such high densities the cell colonies arising from individual protoplasts tend to grow at a fairly early stage in culture. Experiments on somatic hybridisation and mutagenesis, however, require cloning of individual cells. This can only be achieved by plating protoplasts at low densities (100-500 protoplasts/ml). At low density the development process of individual protoplasts, or heterokaryons, can be easily monitored, thereby enabling isolation and identification of hybrid colonies in the absence of a stringent selection system.
The nutritional components of the most commonly used culture media are not sufficient to induce divisions in protoplasts plated at low density.
Kao and Michayluk (1975) developed a complex protoplast culture medium (KM 8p) in which individually cultured protoplasts (Vicia hajastana) are capable of dividing until callus formation. This medium also induced faster divisions in mesophyll protoplasts of alfalfa, pea, potato, and potato + tomato fusion products plated at low densities. The cultures in this medium are placed in darkness since 8p medium turns phytotoxic under strong light.

I Feeder Layer Technique

Another approach to culture protoplasts at low density is the feeder layer technique. Raveh et al. (1973) prepared a feeder cell layer by exposing tobacco cell suspension protoplasts (106 cells ml-1) to an X-ray dose of 2x 103 R, which inhibited division of cells but allowed them to remain metabolically active. Irradiated protoplasts were washed three times to remove toxic substances due to irradiation and then plated in soft agar medium at a density 2.4 x 104/ml. Non-irradiated protoplasts of low density (10-100 protoplasts/ml) were plated over this feeder layer.

II Co-culture Of Protoplasts

Protoplasts from two different species have also been co-cultured to promote their growth or that of the hybrid cells. Metabolically active and dividing protoplasts of two types are mixed in a liquid medium and plated together so that there is cross-feeding between the two types. This enables the protoplasts or cells at low density to undergo sustained divisions. The co-culture method is generally used where calli arising from two types of protoplasts can be morphologically distinguished. For example, mechanically isolated hybrid cells co-cultured with protoplasts isolated from an albino strain will develop green colonies that are readily distinguishable from non-green colonies of albino types (Menczel et at. 1978).

III Microdrop Culture

Microdrop culture has been successfully used to culture hybrid cells of Nicotiana glauca (+) Glycine max (Kao 1977) and Arabidopsis thaliana (+) Brassica campestris (Gleba and Hoffmann 1979). The technique requires specially designed Cuprak dishes which have a smaller outer chamber and a large inner chamber. The inner chamber carries numerous numbered wells, each with a capacity for 0.25-25 u1 droplet of nutrient medium. Individual protoplasts or heterokaryons per 0.25-25 u1 droplet of nutrient medium are transferred by Drummond pipette to each well of the inner chamber of the Cuprak dish. The outer chamber is filled with sterile water to maintain humidity inside the dish. After covering it with a lid, the dish is sealed with parafilm and maintained at optimal light and temperature conditions. The size of the droplet is critical for division of a single protoplast or heterokaryon, as it gives a ratio of cell/ volume of culture medium equal to a cell density 2-4 x 10 3/ml. An increase in the size of the droplet would decrease the effective plating density.

Protoplast Regeneration

Formation of Cell Wall

The process of cell-wall formation may be completed in two to several days although protoplasts in culture generally start regenerating a cell wall within a few hours after isolation. Protoplasts lose their characteristic spherical shape once the wall formation is complete. The regeneration of cell wall can be demonstrated using Calcafluor White ST (American Cyanamide Co., USA) fluorescent stain. The presence of a wall can be tested by incubating protoplasts in 0.1 or 0.01% Calcafluor solution, in an appropriate osmotic stabiliser, for 5 min. The protoplasts are then washed to remove excess dye and mounted on a slide in an osmotically suitable medium. Calcafluor binds to the wall material and fluoresces under a mercury vapour lamp with an excitation filter BG12 and Suppression filter K510. Tinapol solution (Geigy, UK Ltd.) behaves in a manner similar to Calcafluor.

The freshly formed cell wall is composed of loosely arranged microfibrils, the process requiring an exogenous supply of a readily metabolised carbon source (sucrose) in the nutrient medium. Ionic osmotic stabilisers in the medium are reported to suppress the development of a proper wall.

There is a direct relationship between wall formation and cell divisions. Protoplasts with a poorly developed wall often show budding and those which are not able to regenerate a proper wall fail to undergo normal mitosis.

Development of Callus/Whole Plant

Soon after the formation of a wall around the protoplasts, the reconstituted cells show considerable increase in size and first divisions generally occur within a week. Subsequent divisions give rise to small cell colonies. After 2-3 weeks, macroscopic colonies are formed which can be transferred to an osmotic-free medium to develop a callus. The callus may be induced to undergo organogenic differentiation, or whole plant regeneration, following the procedures described in earlier Lessons.

Since the first report (Takebe et at 1971) of plant regeneration from isolated protoplasts, the list of species exhibiting this potentiality has increased. Initially, the majority of taxa which yielded protoplasts capable of regenerating callus or whole plants belonged to Solanaceae but subsequent advances in protoplast technology resulted in differentiation of shoots or plants from isolated protoplasts of several other families, including forage legumes, fibre or pulp crops, various tree species, and even cereals, such as pennisetum, rice and wheat.

Conclusion

Protoplasts provide excellent opportunities for research on plant improvement. To achieve this, protoplast isolation is done which involves the use of several enzymes. These enzymes are available commercially and this has enabled to isolate protoplasts practically from every plant tissue in which cells have not acquired lignification. However, the reproducible potential for regeneration has been observed mostly in protoplasts isolated from leaf mesophyll tissues or cell suspension cultures. This Lesson thus deals with the commonly used protocols for culture and regeneration of plant protoplasts. These include different plating techniques for culture of protoplasts.

Questions

1. Discuss the culture requirements and techniques of protoplast culture.
2. How does the osmoticum and cultural requirements affect protoplast culture?

Note